THE UNIVERSITY OF ALBERTA

IDENTIFICATION OF A GENETICALLY UNSTABLE STRAIN OF *NEUROSPORA CRASSA* AS A MUTANT AT THE *PHEN*-1 LOCUS

BY

KRISHNA KUMAR JHA

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA
AUGUST, 1964

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Identification of a genetically unstable strain of *Neurospora crassa* as a mutant at the *phen*-1 locus" submitted by Krishna Kumar Jha in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

An auxotrophic strain of Neurospora crassa (UA119), isolated after nitrous acid treatment of conidia, was earlier reported to be mutant at the tryp-3 locus. Analysis of the crosses between UA119 and fluffy show the location of the "tryptophan" mutation on the linkage group I, in the vicinity of sex. The allelic nature of the mutation in UA119 with two previously described phen-1 mutants is confirmed by the low frequency of prototrophs in the progeny of crosses with the latter and by the nutritional characteristics of UA119 (any one of the aromatic amino acids or leucine or ethyl acetoacetate is required for growth by phen-1 mutants). The viability of ascospores from "inter-allelic" crosses is low (15-20%) and ascospore isolates with modified nutritional characteristics such as inability to grow on phenylalanine-supplemented medium or a specific requirement for leucine, are frequent. Genetic instability and leakiness are characteristics exhibited by all the three phen-1 mutants but the frequency of phenotypic reversions in UA119 is very high (approx. 10 prototrophs/10^5 viable conidia; colonies larger than 2 mm. on sorbose-agar medium after 3 days at 30 C. counted). The viability of conidia of the phen-1 mutants is reduced to 20% or less by L-phenylalanine, L-leucine or ethyl acetoacetate in sorbose-agar medium, whereas on L-tryptophan- or L-tyrosine-supplemented medium the viability ranges from 40 to 60 percent. The reasons for the apparent scarcity of phen-1 mutants are discussed and it is suggested that ethyl acetoacetate is the best selective compound for the

ABSTRACT

An auxotrophic strain of *Neurospora crassa* (UA119), isolated after nitrous acid treatment of conidia, was earlier reported to be mutant at the *tryp-3* locus. Analysis of the crosses between UA119 and *fluffy* show the location of the "tryptophan" mutation on the linkage group I, in the vicinity of *sex*. The allelic nature of the mutation in UA119 with two previously described *phen-1* mutants is confirmed by the low frequency of prototrophs in the progeny of crosses with the latter and by the nutritional characteristics of UA119 (any one of the aromatic amino acids or leucine or ethyl acetoacetate is required for growth by *phen-1* mutants). The viability of ascospores from "inter-allelic" crosses is low (15-20%) and ascospores isolated with modified nutritional characteristics such as inability to grow on phenylalanine-supplemented medium or a specific requirement for leucine, are frequent. Genetic instability and leakiness are characteristics exhibited by all the three *phen-1* mutants but the frequency of phenotypic reversions in UA119 is very high (approx. 10 prototrophs/10^5 viable conidia; colonies larger than 2 mm. on sorbose-agar medium after 3 days at 30 C. counted). The viability of conidia of the *phen-1* mutants is reduced to 20% or less by L-phenylalanine, L-leucine or ethyl acetoacetate in sorbose-agar medium, whereas on L-tryptophan- or L-tyrosine-supplemented medium the viability ranges from 40 to 60 percent. The reasons for the apparent scarcity of *phen-1* mutants are discussed and it is suggested that ethyl acetoacetate is the best selective compound for the isolation of phen-1 mutants. Various hypotheses with respect to the nature and the primary role of the mutant phen-1 gene are considered and it is suggested that the activity of a transaminase involved in the metabolism of phenylalanine, tyrosine and leucine might be altered as a result of mutation at the phen-1 locus.

ACKNOWLEDGEMENTS

The study was carried out with the guidance and encouragement of Dr. J. Weijer, to whom sincere thanks are expressed. Thanks are also expressed to Dr. W.E. Smith for his suggestions and criticism of the manuscript. Mr. P.O.W. Rhynas and Mr. J. Buffel were kindly helpful in many ways. Without the constant encouragement of my wife Raj the study would not have been possible.

A National Research Council of Canada Scholarship is gratefully acknowledged. The study was also supported by grants from the National Research Council to Dr. J. Weijer.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

INTRODUCTION

Genetic control of specific biochemical reactions was recognized by Garrod (1909) in his studies of "inborn errors of metabolism." Later studies of genetic control of anthocyanins in plants and the eye pigments in *Drosophila* also suggested that genes might act by regulation of specific reactions in a living organism (Beadle, 1959). It was, however, the recognition of *Neurospora crassa* as a suitable organism for induction of nutritional mutations as well as for genetic studies (already demonstrated by Dodge and Lindegren) which made it possible for Beadle and Tatum (1941) to show that mutation in a single gene could stop the biosynthesis of an essential compound. Isolation and study of numerous nutritional mutants in *Neurospora* (Beadle, 1945) and the application of the mutational technique to bacteria (Tatum, 1946) strengthened the concept that a gene determined the synthesis of an enzyme which catalyzed a specific biochemical reaction. Mitchell and Lein (1948) were able to show that a mutant of *Neurospora* unable to synthesize tryptophan was deficient in the activity of an essential enzyme. Studies of several mutants unable to synthesize an essential compound also proved that several genes controlled the various steps of its biosynthetic pathway. Mutants of micro-organisms have helped to outline, in more or less detail, the numerous metabolic pathways involved in the biosynthesis of essential compounds such as amino acids, nucleic acid bases and vitamins (Wagner and Mitchell,1964).

Studies have shown that mutations in some genes result in the loss of activity of a single enzyme (Fincham, 1960). In many cases the mutation causes the production of altered enzyme molecules and these cases have given rise to the concept of the

structural gene - a genetic unit which carries the specificity for determining the structure of a particular enzyme. The demonstration that an enzyme may consist of separable and different polypeptide subunits, each specified by distinct regions of the genetic material (Yanofsky _et al_., 1961), has modified the earlier "one gene - one enzyme" hypothesis into the concept of "one gene - one polypeptide". In fact, the co-linearity of a gene with the polypeptide structure specified by it, has been demonstrated (Yanofsky _et al_., 1964; Sarabhai _et al_., 1964).

The synthesis and the activity of an enzyme is dependent not only upon the gene or genes which specify the structure of the component protein but also upon the activity of other genes and the nature of intracellular environment which again is partially genetically determined. The studies of genetic control of induction and repression of enzyme synthesis in bacteria (Jacob and Monod, 1961) have given rise to another significant concept which recognizes a distinct class of genes termed the "regulatory genes". According to this concept, a regulatory gene does not specify the structure and properties of an enzyme but controls the rate of synthesis of enzyme molecules. The product of the regulatory gene, which may be a protein (Stent, 1964), in combination with some specific small molecule, alters the rate of synthesis of an enzyme or a group of enzymes.

A mutation can lead to very complex phenotypic effects due to alteration of the metabolic pattern. Even in the case of a nutritional mutant exogenous supply of the deficient nutrient does not completely restore the premutation or "wild type" phenotype. It is necessary to distinguish, if possible, the primary effects of a gene mutation from the numerous physiological and

morphological effects which may have resulted due to inter-relationships of many metabolic pathways. These inter-relationships themselves, however, may be obscure or unknown till emphasized as a result of mutations.

Mutations in Neurospora crassa at the locus designated phen-1 on linkage group I (phen refers to phenylalanine) provide an interesting example of unsuspected metabolic relationships. These mutations are the subject of the present study. The characteristic of the phen-1 mutant strains is their ability to grow only in the presence of one of the aromatic amino acids or leucine or ethyl acetoacetate or a number of related substances. The nature and the mechanism of expression of the mutant gene are of interest because the biosynthetic pathways of the aromatic amino acids are quite separate from the biosynthetic pathway of leucine.

The main purpose of the experiments reported in this thesis was to establish the genetic location of the mutation in a strain of Neurospora crassa. Though Leeuw (1963) reported it to be located at the tryp-3 locus on linkage group II, no actual data were supplied. As a "tryp-3" mutant it was of interest to the present author because of its high rate of apparent reversions and some response to indole supplement. In addition to the data which identifies the strain (designated as UA119) as a phen-1 mutant, the frequency of reversion in UA119 as compared to the frequencies in two other phen-1 strains (previously described by Barratt and Ogata, 1954) are also reported. The effect of different supplements on the viability of conidia of phen-1 mutants is described. The genetic nature and the hypotheses which may explain the nutritional characteristics of phen-1 mutants and their "modified" derivatives are discussed.

REVIEW OF LITERATURE

1. Studies with phen-1 mutants

Hungate and Mannell (1952), in an investigation of the mutagenic action of radioactive sulphur, obtained certain mutant strains of Neurospora Crassa which were found to grow when provided with either leucine or some other amino acids. Two of these strains were later studied by Barratt and Ogata (1954).

The latter authors tested a large number of compounds for growth-promoting activity using auxanographic methods. The compounds found to be most active were DL- or L-leucine, DL-norleucine, DL- or L-phenylalanine, phenylpyruvic acid, DL- or L-tyrosine, ethyl acetoacetate, L-tryptophan, β-indolepyruvic acid, indole and kynurenine. The following compounds were found to be less active than the above group but promoted some growth: DL-valine, DL-threonine, ρ-hydroxyphenylpyruvic acid, butyric acid, α-keto butyric acid, D-leucine and α-amino butyric acid. In addition DL-homoserine, dihydroxyisoleucine, caproic acid and DL-norvaline were found to be slightly stimulatory but significantly less than the previously mentioned compounds. Quantitative growth tests were made in flask assays and growth tubes and it was found that on molar basis, phenylalanine was the most active compound followed in decreasing order of activity by tryptophan, tyrosine, leucine, norleucine and ethyl acetoacetate. Barratt and Ogata (1954) found that the D- isomers of the amino acids were inactive by themselves but could be utilized in the presence of the corresponding L- isomers. The phen strain H6196 required essentially the same amounts of phenylalanine or leucine as the standard strains known to be blocked in

phenylalanine or leucine synthesis. With limiting amounts of a supplement, either in flasks or in growth tubes, the mycelial weight or the rate of growth respectively was found to be proportional to the amount of supplement. The growth factor requirements for the phen strain were not eliminated by altering the level of trace elements, addition of a chelating agent or changes in the pH of the medium. It was found that isovaleric acid and homogentisic acid, (known to be intermediates between leucine or tyrosine and acetoacetic acid in ketogenesis in rat liver) were inactive in supporting the growth of phen strain of Neurospora. Barratt and Ogata (1954) in their conclusion proposed the hypothesis that a mutation in the phen locus results in the accumulation of some inhibitor, the action of which is reversed by the growth-promoting compounds.

Barratt and Ogata (1954) also determined the linkage relationships of the phen locus. The mutation in H6196 segregated as a single locus in crosses with wild-type (prototrophic) strains and it was located on the left arm of linkage group I, 3.4 units from centromere and proximal to sex. Two other strains (H3791 and Y31032) were found to be probably mutant at the same locus on the basis of negative complementation tests with H6196 and because of similar response to leucine, an aromatic amino acid or ethyl acetoacetate. The mutant locus in H3791 was found to be closely linked to sex and among 20 ascospores from a cross between H3791 and H6196 no prototrophs were found.

The possibility that phenylalanine and leucine may be interconverted via acetoacetate was tested by Barratt, Fuller and Tanenbaum (1956) using DL-phenylalanine and DL-leucine, both labelled with radioactive carbon (β position). Only a small

percentage of the initial radioactivity was found in the respiratory carbon dioxide. There was no significant difference between the radioactive distribution pattern among the amino acids from the phen strain (H6196) when compared with the leu-1 and arom strains also grown on equivalent amounts of the labelled supplement. In the phen strain endogenous synthesis of both phenylalanine and leucine occurred irrespective of the compound supplied. The "80 percent ethanol" fraction from the phen strain mycelium contained two interesting ninhydrin-negative compounds when either phenylalanine or leucine was used as a supplement. These compounds were not present in the extracts of the wild type. The authors considered these unidentified compounds as normal intermediates in the catabolic pathways of these amino acids but suggested that these compounds may give a clue to the nature of some unknown compound required by phen strains. The changes in the amino acid composition of the mutant strain grown in minimal medium when compared with the wild type, are not known at present.

Recently Newmeyer (1963) in a short note mentions the failure of many phen (H6196) isolates from certain crosses to grow well on phenylalanine-supplemented medium containing sucrose as a carbon source. Although unable to grow on minimal medium, these isolates have an altered phenotype when compared to the parental phen strain; they respond slowly to phenylalanine but in leucine-supplemented medium they grow rapidly. The growth of these "slow" phen strains on ethyl acetoacetate is not impaired. Newmeyer finds that both "fast" and "slow" phen isolates grow equally well on phenylalanine-supplemented medium when sucrose is replaced by glycerol as a carbon source. She suggests that the difference between "fast" and "slow" phen isolates exists in

their relative sensitivity to sucrose. The genetic basis of the phenotypic modification has not been determined.

2. Studies with other mutants in which the growth inhibition is relieved by more than one amino acid.

Analysis of mutants which have a simultaneous requirement for a number of compounds, such as the *aromatic* mutants or the *isoleucine* - *valine* mutants, is interesting since these mutants indicate a common origin of the deficient compounds or a common enzyme system. Mutants, on the other hand, whose requirements are met by any one of a number of compounds indicate metabolic relationships which may not be obvious otherwise. *Phen*-1 mutation is an example of the latter type. A review of studies with other mutants, similar to *phen*-1 mutants in so far as their growth is promoted by one of several compounds, is of interest since these studies point to the genetic and physiological aspects which should be kept in view.

(a) Proline - Arginine relationships in mutants of *Neurospora crassa*.

The mutants called proline-2, proline-3 and proline-4 (the latter two are also called *arg*-8 and *arg*-9 respectively) were found to be leaky i.e. they grew slowly on minimal medium (Mitchell and Mitchell,1952). The growth of these mutants, however, was stimulated by proline, arginine and arginine precursors, ornithine and citrulline. Mitchell and Mitchell (1952) showed that a common suppressor mutation in combination with any of the non-allelic *prol*-2, *prol*-3 or *prol*-4 alleviated the proline requirement. The work of Vogel and coworkers (see Vogel,

1955) has shown that exogenous ornithine is readily converted to proline. It was found that endogenous ornithine did not contribute to the synthesis of proline. Though glutamic γ-semialdehyde is a common precursor for proline and arginine, physical separation of pathways for proline and for arginine synthesis was indicated (Vogel 1955). It was suggested that glutamic γ-semialdehyde as a precursor for ornithine synthesis is not available for proline formation. However, when exogenous ornithine is available, it is converted to glutamic γ-semialdehyde which is utilized for proline biosynthesis. The studies of Vogel and Kopac (1959) supported the earlier view of Vogel (1955) that the pathways of proline and arginine biosynthesis are physically separated. Moreover, Vogel and Kopac (1959) obtained evidence that a proline-requiring strain (35401) in addition could utilize pentahomoserine (α-amino-δ-hydroxyvaleric acid) and α-keto-δ-amino-valeric acid for the synthesis of proline, though these compounds are not involved in the normal route of proline formation (via glutamic acid and glutamic γ-semialdehyde). Similar relationships between proline and ornithine have been found in Escherichia coli and Torulopsis utilis (Vogel 1955).

Davis and Thwaites (1963) have shown that the strains which carry the suppressor mutation (called S or arg-12) are low in the activity of the enzyme Ornithine Transcarbamylase. The suppressor locus was found to be the structural gene for this enzyme. Davis (1962) explained the action of this suppressor mutation which relieves the proline requirement (the same suppressor also relieves the pyrimidine requirement created by certain mutations for the pyrimidine pathway) by assuming that since it

reduces the rate of utilization of ornithine, adequate glutamic γ-semialdehyde is made available for the formation of proline. The role of arginine and citrulline in removing proline requirement in these strains may be explained on the basis of the ornithine cycle operational in <u>Neurospora crassa</u> as shown by Srb and Horowitz (1944). The fact that glutamic γ-semialdehyde is a common precursor for proline and arginine synthesis, suggests that arginine could also possibly act by feedback inhibition and repression of the enzyme systems involved in the arginine pathway and thus allow glutamic γ-semialdehyde to be available for proline biosynthesis. The role of feedback inhibition and repression by the end product has become very significant in the regulation of metabolic pathways in micro-organisms (Umbarger, 1961; Vogel 1961).

(b) Threonine-Isoleucine relationship in a mutant of <u>Neurospora crassa</u>

The mutant <u>thr</u>-1 (46003) is able to make appreciable growth in the absence of added metabolites. For vigorous growth, however, it needs any one of the following compounds: threonine, homoserine, α-aminobutyric acid, α-ketobutyric acid, isoleucine or the α-keto analogue of isoleucine (Emerson, 1950). Homoserine is a precursor for threonine and methionine biosynthesis. It was pointed out by Teas (see Discussion following the paper by Emerson, 1950) that the mutant 46003 does not have a methionine requirement but can grow in the presence of homoserine. He suggested that in this strain the genetic block existed in the biosynthesis of homoserine and that α-aminobutyric acid and isoleucine are both able to give rise to threonine through the synthesis of some precursor.

However, isoleucine is derived from α-keto-butyric

acid which is a product of deamination of threonine (Umbarger and Davis 1961). Amination of α-ketobutyric acid gives rise to α-aminobutyric acid. In Escherichia coli the enzyme Threonine Deaminase had been found to be specifically and competitively inhibited by L-isoleucine, thus providing a negative feedback mechanism controlling the biosynthesis of isoleucine (Umbarger 1956). Hence the idea of Teas (1950) that isoleucine, ketobutyrate or α-aminobutyrate may act as alternate precursors for threonine does not seem likely. While homoserine is a precursor and thus may provide increased amounts of threonine, it seems more plausible that exogenous isoleucine, α-ketobutyrate or α-aminobutyrate have a sparing effect on the endogenous threonine. Isoleucine, for example, by inhibition and repression of the enzyme Threonine Deaminase would reduce the conversion of threonine to α-ketobutyrate necessary for the endogenous synthesis of isoleucine.

(c) Mutant strains requiring tryptophan or nicotinic acid (the nt mutants).

The nt mutant strains are particularly interesting in relation to phen-1 strains since the studies of nt mutant strains have shown the influence of the genotype i.e. presence or absence of "modifiers" on the phenotypic expression of the nt allele. Mutation at the nt locus creates a nutritional requirement which can be satisfied by nicotinic acid, tryptophan or other related compounds.

Nicotinic acid is derived from tryptophan in Neurospora crassa and Partridge, Bonner and Yanofsky (1952) were able to show that in a nt strain tryptophan was converted to nicotinic acid. They were also able to prove that though the nt mutant

did not grow on anthranilic acid alone, anthranilic acid was converted to tryptophan, in the presence of nicotinic acid. Bonner, Yanofsky and Partridge (1952) demonstrated that the _nt_ mutant synthesized considerable amounts of tryptophan even though it was unable to grow unless indole, tryptophan or nicotinic acid was available in the medium. As the strain did not grow in the presence of anthranilic acid, these authors suggested that the _nt_ strain was blocked in the conversion of anthranilic acid to indole (indole was believed to be a precursor for tryptophan at that time).

The studies of Haskins and Mitchell (1952) and Newmeyer and Tatum (1953), however, showed that the position of the so-called "genetic block" was dependent upon the genotype of the particular _nt_ strains. Haskins and Mitchell (1952) could classify some original isolates and "modified" _nt_ strains i.e. those derived from crosses between the original _nt_ mutants and a wild type or from crosses between _nt_ mutants themselves, into four classes. Type 1, represented by the strain C86, could grow in the presence of any one of the following compounds: phenylalanine, anthranilic acid, tryptophan and nicotinamide. Type 4 represented the other extreme; the strains in this category grew well when provided with nicotinic acid and grew only a little in the presence of tryptophan. The type 4 strains did not grow at all when provided with either phenylalanine or anthranilic acid as a supplement. All the four types were uniform in two characteristics i.e. they did not grow on minimal and all grew well when nicotinic acid was available. The presence of six different "modifying" genes in various combinations with a particular _nt_ allele produced phenotypes which differed in

their response to various supplements.

Newmeyer and Tatum (1953) discovered a similar situation in studies with nt strains. These authors found, in addition to the influence of the genotype, that external factors such as the presence of p-aminobenzoic acid in the culture fluid, modified the phenotypic response of a nt strain. The strain 65001 a, for example, could grow on anthranilic acid when also provided with p-aminobenzoic acid, though it did not grow when either compound was available alone. As also noted earlier by Haskins and Mitchell (1952), the strain C86 could use anthranilic acid in the absence of exogenous p-aminobenzoic acid.

The nature of gene action and the metabolic pattern in the nt strains remains unknown. Newmeyer and Tatum (1953) have suggested a number of possibilities but experimental proof for any one is lacking at this moment.

3. Metabolism of the aromatic amino acids, leucine and acetoacetate.

(a) Biosynthesis of aromatic amino acids.

The subject of biosynthetic pathways of aromatic amino acids in micro-organisms has been reviewed in detail by Umbarger and Davis (1961). Most of the information has been derived from investigations in *Escherichia coli* and *Aerobacter aerogenes*. From mutations in *Neurospora* it has become evident that the biosynthetic pathways of the aromatic amino acids are the same in this organism as in the bacteria. Phenylalanine, tyrosine, tryptophan and p-aminobenzoic acid are derived from a common precursor, chorismic acid (Gibson and Jackman, 1963; Gibson,

1964; Gibson and Gibson 1964; Gibson, Gibson and Cox, 1964). Mutations which block any one of the steps in the pathway common to all the aromatic compounds, create a nutritional requirement for all of the compounds. The first specific precursors for the aromatic compounds are erythrose 4-phosphate and phosphoenolpyruvate, which give rise to 3-deoxy-D-arabinoheptulosonic acid 7-phosphate, a seven carbon compound. The latter is converted to 5-dehydroquinic acid. The subsequent intermediates in the pathway are 5-dehydroshikimic acid, shikimic acid, shikimic 5-phosphate and chorismic acid. The intermediates following 3-deoxy-D-arabinoheptulosonic acid 7-phosphate including chorismic acid are non-aromatic ring compounds. Chorismic acid gives rise to prephenic acid which yields the α-keto precursors phenylpyruvic acid and p-hydroxyphenylpyruvic acid. The latter by a transamination reaction are converted to phenylalanine and tyrosine respectively. The first specific step in tryptophan biosynthesis involves aromatization of chorismic acid and the introduction of an amino group from glutamine to yield anthranilic acid. Chorismic acid is also converted to p-aminobenzoic acid by a specific pathway in which the nitrogen donor also appears to be glutamine. On the basis of enzymatic studies in wild type and mutant strains Gross and Fein (1960) have shown that the common pathway up to shikimic acid is the same in _Neurospora crassa_. Mutants are also known which accumulate but cannot utilize shikimic acid. Presumably these mutants are unable to carry out the reactions following the synthesis of shikimic acid. Mutant strains which are specifically blocked in the biosynthesis of a particular compound and do not have a multiple requirement are also known. It should be mentioned

that Barratt and Ogata (1954) found shikimic acid to be inactive in promoting growth of phen-1 strains.

Not much is known about the mechanisms of regulation of the common pathway as well as of the specific biosynthetic pathways since the end product of the common aromatic pathway has been discovered very recently. Morgan, Gibson and Gibson (1963) found that addition of L-tryptophan to a cell free system capable of forming anthranilate from shikimic acid inhibited the synthesis of anthranilic acid (feedback inhibition) and stimulated the production of phenylpyruvic acid and p-hydroxyphenylpyruvic acid. Lester (1963) showed that the phenomena of feedback inhibition and repression were operative in *Neurospora* for the regulation of early reactions in the biosynthesis of tryptophan. The effect of an aromatic amino acid on the metabolism of related amino acids may be significant in the phen-1 strains. It may be mentioned here that p-aminobenzoic acid is unable to stimulate growth of phen-1 strains.

(b) Degradative pathways of aromatic amino acids.

Most of our knowledge concerning the degradative pathways of phenylalanine and tyrosine has been derived from animal rather than from microbial sources. Henderson, Gholson and Dalgliesh (1962) have extensively reviewed the metabolism of aromatic amino acids. Degradation of phenylalanine involves, first, its conversion to tyrosine by a hydroxylation reaction. Barratt, *et al.*, (1956) have shown that conversion of phenylalanine to tyrosine also occurs in *Neurospora crassa*. The degradation of tyrosine involves its conversion to p-hydroxyphenylpyruvic acid, presumably by a transaminase. The α-keto acid via homogentisic acid, maleyl acetoacetate and fumaryl acetoacetate

is finally split into fumarate and acetoacetate. It is interesting to note that the first "inborn error of metabolism" studied by Garrod (1909) was alcaptonuria, a metabolic disorder of man which is characterized by excretion of homogentisic acid. The *phen*-1 mutants are very slightly stimulated by p-hydroxyphenylpyruvic acid and somewhat more by phenylpyruvic acid. Barratt and Ogata (1954) found that homogentisic acid was inactive as a supplement for *phen*-1 strains.

Due to the fact that tryptophan is a precursor of nicotinic acid in animals and *Neurospora crassa*, the metabolism of tryptophan has received a great deal of attention. The subject has been reviewed by Bonner and Yanofsky (1951) and Henderson *et al*. (1962). It is important to note that *phen*-1 strains are able to grow in the presence of kynurenine which is an intermediate in the degradative pathway for tryptophan (Barratt and Ogata 1954). Evidence for the conversion of tryptophan to anthranilic acid vis kynurenine was presented by Haskins and Mitchell (1949). A wild type strain when grown in the presence of high levels of tryptophan excreted anthranilic acid in the medium. A mutant strain which was able to grow on anthranilic acid could also utilize kynurenine. The *phen*-1 strains, however, cannot grow in the presence of anthranilic acid, whereas indole, tryptophan and kynurenine are growth-promoting compounds (Barratt and Ogata 1954).

(c) Biosynthesis and Degradation of Leucine

The biosynthetic pathway for leucine has been recently elucidated in *Neurospora crassa* and *Salmonella typhimurium* (Jungwirth *et al*., 1963; Gross *et al*., 1963; Burns *et al*., 1963; Calvo *et al*., 1962). The intermediates in biosynthesis of

leucine are α-ketoisovalerate, β-carboxy-β-hydroxyisocaproate, α-hydroxy-β-carboxyisocaproate and α-ketoisocaproate. The first intermediate, α-ketoisovalerate, is also the immediate precursor for valine. The final step in the biosynthesis of leucine is amination of α-ketoisocaproate, catalyzed presumably by a transaminase. Information concerning the degradative pathway of leucine in micro-organisms, including _N. crassa_, is rather scanty. The role of leucine in ketogenesis in animal tissues has been recognized for a long time (Coon, Robinson and Bachhawat, 1955). In an outline the pathway consists of conversion of leucine to α-ketoisocaproate which is finally converted to acetoacetate and acetyl CoA. The intermediates between α-keto-isocaproate and acetoacetate and acetyl CoA are isovaleryl CoA, isopentenoyl CoA, β-methylglutaconyl CoA and β-hydroxy-β-methylglutaryl CoA. The prominent feature in this degradative pathway is the role of Coenzyme A. β-hydroxy-β-methylglutaryl CoA is cleaved into acetoacetate and acetyl CoA. Since acetyl CoA can also be condensed to acetoacetate hence one mole of leucine on complete catabolism leads to the formation of 1.5 moles of acetoacetate. An important reaction of β-hydroxy-β-methylglutaryl CoA is its conversion to mevalonic acid which is the source of the isoprene units in β-carotene and other carotenoids (Durr and Rudney, 1960). Biosynthesis of cholesterol has been reviewed by Popjack and Cornforth (1960). Braithwaite and Goodwin (1960) have shown that the radioactive labels from leucine were incorporated into carotene by _Phycomyces blakesleeanus_.

(d) Metabolism of acetoacetate

There is no specific information available about the role of acetoacetate in Neurospora except of course the fact that phen-1 mutants are able to grow in the presence of ethyl acetoacetate. As pointed out earlier, acetoacetate can arise from degradation of tyrosine and leucine. In animals the production of acetoacetate has received a great deal of attention because accumulation of acetoacetate is a metabolic disorder known as ketosis. It has been recognized that acetoacetate is a normal product of fatty acid oxidation and that it can be oxidized in all tissues except the liver. The enzymatic mechanizms of fatty acid oxidation have been reviewed in detail by Green and Wakil (1960). Oxidation of fatty acids yields acetoacetyl CoA and acetyl CoA.

Acetoacetyl CoA is reversibly converted to acetyl CoA by the enzyme β-ketoacyl CoA thiolase. Acetoacetyl CoA in the presence of acetyl CoA is also converted to acetoacetate and free coenzyme A. It is interesting to note that the conversion of acetoacetyl CoA to acetoacetate perhaps involves condensation of acetoacetyl CoA with acetyl CoA to produce β-hydroxy-β-methylglutaryl CoA. The latter is then cleaved to acetoacetate and acetyl CoA and free coenzyme A. Hence, an interrelationship between fatty acid oxidation, the degradation of leucine and the biosynthesis of carotenoids is seen to involve the key intermediate, β-hydroxy-β-methylglutaryl CoA.

4. Transaminases in *Neurospora crassa*

A brief review of the studies on transamination activities in Neurospora is justified because of some growth promoting activity of phenylpyruvic acid and p-hydroxyphenylpyruvic acid and the inability of α-ketoisocaproic acid to stimulate growth of *phen*-1 strains. As mentioned earlier, the final step in the biosynthesis of phenylalanine, tyrosine and leucine is a transamination reaction. Transamination is again the first step in the degradation of tyrosine and leucine.

Transamination activities in crude and partially purified extracts of *Neurospora* have been examined by Fincham and Boulter (1956), Ames and Horecker (1956), Jacoby and Bonner (1956), Monder and Meister (1958) and Seecof and Wagner (1959a, 1959b).

Ames and Horecker (1956) have reported that the conversion of imidazole acetol phosphate to L-histidinol phosphate, a reaction involved in the biosynthesis of histidine, is catalyzed by a transaminase. The α-keto phosphate ester is converted to α-amino phosphate in the presence of an amino group donor such as glutamate, L-α-aminoadipate, L-arginine or L-histidine. Glutamate was the most effective amino donor.

Monder and Meister (1958) prepared extracts of *Neurospora* which transaminated glutamine and α-ketosuccinamate to yield asparagine and α-ketoglutaramate.

The report of Jacoby and Bonner (1956) that a transaminase from *Neurospora* catalyzed the conversion of kynurenine to kynurenic acid in the presence of a variety of α-keto acids is particularly interesting in view of the fact that kynurenine

promotes the growth of phen-1 mutants. The authors found that pyruvate and α-ketoglutarate were the most efficacious α-keto substrates; phenylpyruvic acid, indolepyruvic acid and α-ketobutyric acid were also active but the rate of transamination was lower than with pyruvate or α-ketoglutarate; the least active keto substrates were α-ketoisovalerate and α-keto-β-methylvaleric acid. The α-keto analogue of leucine was not tested in this study.

Fincham and Boulter (1956) have postulated four different transaminases which can utilize α-ketoglutarate as a substrate. The hypothesis was proposed on the basis of the observed increase in certain transaminase activities when the mycelium was grown in the presence of certain amino acids. Fincham and Boulter (1956) proposed that a single enzyme, or a group of very similar enzymes, catalyzed glutamate formation from phenylalanine, isoleucine, valine, leucine, morleucine, methionine, α-aminobutyric acid and tryptophan, in a decreasing order of activity.

Seecof and Wagner (1959a,b) have partially purified a transaminase which reacted with 13 of the 19 amino acids tested. The α-keto substrates used were α-ketoglutarate, phenylpyruvate, pyruvate, α-ketoisovalerate, and α-keto-β-methylvalerate. The enzyme was relatively most active with phenylalanine as donor and α-ketoisovalerate, α-keto-β-methylvalerate or α-ketoglutarate as amino group recipients. With phenylpyruvate as amino group recipient, isoleucine, valine and leucine were efficient amino group donors. The enzyme did not react with ornithine, alanine or aspartate. Glutamate was utilized relatively

less efficiently by the enzyme in the amination of phenylpyruvate when compared with the branched chain amino acids. The question whether or not the enzyme preparation was very active in the transamination reaction between α-ketoisocaproate and glutamate as compared to other amino group donor was not investigated. Qualitatively the preparation was found to be active in the amino group transfer between leucine and α-ketoglutarate.

Genetic control of transaminases has not been reported so far in _Neurospora crassa_. At least in one case (Seecof and Wagner, 1959a,b), in which transaminase may be involved in the metabolism of a large number of amino acids, the organism may not tolerate the loss of the enzyme. Modifications are, however, possible if the metabolic and physiologic alterations are not too drastic or are rectified by changes in environment.

In _Escherichia coli_ an isoleucine auxotroph was found to be deficient in a transaminase (Rudman and Meister 1953). This enzyme transferred amino groups between glutamate, isoleucine, valine and leucine. The loss of the transaminase resulted in an absolute requirement for isoleucine because other transaminases catalyzed the reactions for the synthesis of leucine and valine. The auxotroph had a partial requirement for valine since the alanine-valine transaminase did not meet the valine requirements for rapid growth.

Accumulation of the α-keto analogues or the inability to utilize them for growth by mutant strains may indicate the loss or modification of a transaminase. Investigation of the transaminase _in vitro_, however, may not explain the observed

phenotypic behaviour. Seecof and Wagner (1959b), for example, found that a mutant strain of *Neurospora* which was inhibited by threonine at high temperature and accumulated the α-keto analogues of isoleucine and valine, possessed a transaminase with the same properties *in vitro* as the wild type enzyme. Wagner *et al*., (1964) have investigated a large number of isoleucine-valine auxotrophs of Neurospora and find that many of the mutant strains are unable to utilize α-keto acids for the synthesis of isoleucine and valine. Examination of the mycelial extracts, however, shows that a transaminase necessary for the reactions is present.

MATERIALS AND METHODS

The mutant strain UA119 was isolated as a tryptophan auxotroph by Leeuw (1963) by the "inositol-less death" selection technique of Lester and Gross (1961). Nitrous acid was used as the mutagenic agent. The experiment in which UA119 was isolated also yielded twelve other tryptophan mutants, two of which were reported to grow actively with indole as a supplement (Leeuw, 1963).

<u>Phen</u>-1 strains H6196 and H3791 (both of mating type a) were obtained from the Fungal Genetics Stock Center, Hanover, New Hamshire. These mutants were isolated by Hungate and Mannell (1952) using radioactive sulphur as a mutagenic agent. The strains have been described by Barratt and Ogata (1954).

<u>Fluffy</u> (strain L, mating type <u>a</u>) was used as a linkage group II marker because it is closely linked to the <u>tryp</u>-3 locus (Barratt, Newmeyer, Perkins and Garnjobst, 1954). A leucine requiring strain (<u>leu</u>-3, allele R156, mating type <u>a</u>) was used as a linkage group I marker.

The minimal medium of Vogel (1956) with one or two percent sucrose was commonly used for vegetative growth in liquid or agar (Bacto-agar 1.5%). The <u>phen</u>-1 strains were maintained on tryptophan supplemented medium (50 mg. L-tryptophan/liter). The strain UA119 requires inositol as an additional supplement. In Ryan Growth Tube assays it was found that at least eight mg. i-inositol/liter were necessary to support vegetative growth in the strain UA119 equal to the rate of growth of wild types on minimal medium. However, it was found that conidiation was improved with 15 to 20 mg. inositol/liter. For convenience the inositol-supplemented

medium is referred to as minimal medium in the text.

Crosses were made on the synthetic medium favouring sexual reproduction (Westergaard and Mitchell, 1947). However, the crosses between UA119 and the phen-1 strains, for the purpose of obtaining prototroph frequencies in large ascospore populations, were made on Bacto Corn Meal Agar containing 0.2% dextrose. The crossing medium in this case was supplemented with 200 mg. L-phenylalanine/liter.

Compounds such as indole, ethyl acetoacetate and the α-keto acids were added as filter-sterilized solutions to appropriate amounts of autoclaved minimal media.

Sorbose-supplemented medium was used in reversion experiments and in ascospore plating experiments. Sorbose, when added to the medium containing low concentrations of sucrose or glucose, causes restricted, colonial growth of Neurospora (Tatum, Barratt and Cutter, 1949). The sorbose-agar medium used in the present study contained 0.1% dextrose and 2% Difco Bacto-agar in addition to the salts and biotin of Vogel medium. Sorbose was always autoclaved separately in aqueous solution and appropriate amounts of the sterilized solution were added to autoclaved agar medium. This procedure prevents dark coloration of the medium due to caramelization.

All the chemicals, including amino acids and other organic compounds, used in this investigation were obtained from commercial sources. The compounds were of the highest available purity and were used without any repurification.

The nutritional characteristics of UA119 and the other phen-1 strains were examined in liquid and agar media. Tests in liquid media were made either in 18 x 150 mm. culture tubes

(10 ml. medium) or in 125 ml. Erlenmeyer flasks (20 ml. medium). Generally a small amount of conidia carried on the tip of the inoculation needle was used as the inoculum but a mycelium-free suspension of conidia was pipetted in appropriate amounts when an inoculum of a known concentration of conidia was desired. It was found necessary to use 10^5 or smaller samples of conidia of UA119 to reduce growth due to reversions. Growth due to reversions could also be taken into consideration when conidia were streaked on the agar medium in petri dishes.

Crosses were made by dusting conidia on the protoperithecial parent, either in petri dishes or on slants in culture tubes. The protoperithecial parent was incubated for four or five days at room temperature (21-22 C) before conidia from five or six days old cultures of the second parent were applied. However, in the crosses between UA119 and the <u>phen</u>-1 strains, when large populations of ascospores were collected, the protoperithecial parent was fertilized by spreading 0.5 ml. of a mycelium-free suspension of conidia. This method gave a large number of well distributed perithecia.

The prototroph recombinants and/or revertants frequencies in the crosses between <u>phen</u>-1 strains were estimated by plating 10^5 or more ascospores from a cross in minimal (i.e. inositol-supplemented) sorbose-agar medium. The viability of ascospores was estimated by spreading a known number of ascospores in sorbose-agar medium (supplemented with 50 mg. L-tryptophan and 100 mg. L-leucine per liter) in petri dishes. The spores were collected four weeks after fertilization from petri dishes kept at 21-22 C. All the perithecia as well as ejected ascospores were collected in 20-40 ml. of distilled

water. The perithecia were squeezed open with forceps and the entire mixture was vigorously shaken in a screw-cap bottle. Filtration through a double layer of surgical gauze gave a heavy suspension of ascospores. The suspension was centrifuged at low speed for a few minutes and the spores were washed with a 1% solution of a commercial sodium hypolchlorite disinfectant (Javex). The washed spores were centrifuged and suspended in sterile distilled water. The concentration was estimated by counting the number of spores in 0.1 ml. aliquots spread on a glass slide. This method was found to be more reliable as compared with counts in a blood counting chamber. Twenty ml. of the suspension were added to an autoclaved non-solidified sorbose-agar medium kept at 60° C. in a water bath. After 30 minutes at this temperature, five ml. aliquots were spread on a layer of minimal sorbose-agar. The plates were incubated at 30° C.

In other crosses, random ascospores were individually isolated by the usual technique and were transferred to small tubes containing minimal medium supplemented with the aromatic amino acids (each at 50 mg./liter), L-leucine (100 mg./liter) and ethyl acetoacetate (100 mg./liter).

For estimation of reversion frequencies, conidia from six days old slants were suspended in sterile distilled water, filtered through glass wool, centrifuged and resuspended in sterile distilled water. Conidial concentrations were calculated using the Bright-line Hemocytometer and adjusted to the desired concentrations. An aliquot containing the desired number of conidia was added to non-solidified minimal sorbose-agar medium kept at 45° C. The medium was then distributed uniformly to a

number of plates (approximately seven or eight plates per 100 ml. medium). Each plate contained approximately 10^5 conidia; higher density was avoided because of the slight leakiness of the *phen*-1 strains. The plates were incubated at 30° C. For estimation of viability of conidia in a particular medium, 0.1 ml. of a suspension containing 10^3 conidia per ml. was spread on sorbose-agar. Reversion frequencies were estimated on the basis of viability of conidia on sorbose-agar medium supplemented with 50 ml. L-tryptophan per liter.

RESULTS AND DISCUSSION

I. Location of the "tryptophan" mutation in UA119 on linkage group I.

A cross was made between UA119 and _fluffy_ (mating type _a_), with the latter as the protoperithecial parent. Seventy-five viable ascospores which had been ejected from perithecia were selected by plating an ascospore suspension on sorbose-agar medium supplemented with L-tryptophan. The data (see Table I) indicated that the "tryptophan" mutation in UA119 was not linked to the gene for _fluffy_. There was a deficiency of _fluffy_ isolates in this sample and among the conidial (non-fluffy) isolates there was an excess of prototrophs (termed "wild" in Table I). The strain UA119 is also inositol-deficient, therefore the segregation for the inositol (_inos._) allele was determined among the "conidial" ascospore colonies. The segregation for _inos_, which is located on linkage group V, was found to be 24 $inos^+$: 26 $inos^-$. The deficiency of the auxotrophic class (tryptophan requiring) was therefore believed to be due to low viability of ascospores of this class and to the method employed for isolation of ascospores

TABLE I

Segregation of "fluffy" and "auxotroph" phenotypes in a cross between UA119 and "fluffy" tester strain.

Phenotype	No. of ascospores
conidial, wild*	41
conidial, auxotroph*	9
fluffy, wild	14
fluffy, auxotroph	11
Total	75

* Wild types do not require tryptophan; auxotrophs require tryptophan.

The cross between fluffy and UA119 was repeated and 600 ascospores (viability 77%) were individually isolated at random from the population ejected out of perithecia. Segregation for fluffy and tryptophan-requirement was observed among 466 ascospores which germinated. It was also noted at this time that UA119 had a relatively reduced amount of conidiation and distinctly pale colour when compared with the parent strain 89601 or other wild types. The segregants from the cross fluffy, a x UA119, A could be classified into the following morphological classes:

(1) growth, conidiation and colour resembling the wild types;

(2) poor growth and conidiation and distinctly pale in colour;

(3) fluffy (aconidial) morphology and vigorous growth;

(4) fluffy morphology but poor growth.

The data given in Table II show that the morphogical features were very closely associated with the nutritional characteristics of the cultures. There were, however, a few conidial isolates which resembled, morphologically, class 1 but were auxotrophic, whereas others which resembled class 2 but were found not to be tryptophan requiring. Not all of the fluffy isolates could be classified for nutritional characteristics but the vigorously growing fluffy strains were found all to be prototrophic and those poorly growing fluffy isolates which were tested were all auxotrophic.

The results confirmed that the "tryptophan" mutation in UA119 was not linked with fluffy and consequently could not be located at the tryp-3 locus.

TABLE II

Segregation of _fluffy_ and auxotrophy in the second cross between UA119 and _fluffy_ (random ascospore analysis).

Class	Morphology	Growth in minimal medium	Number of ascospores
1	Growth and conidition vigorous; colour deep	+	127
2	Reduced growth and conidiation; pale colour	-	115
3	same as class 1	-	3
4	same as class 2	+	7
5	fluffy; growth vigorous or reduced	+ or -	214
		Total	466

The segregation data obtained from the initial cross between UA119 and _fluffy_ had shown that the mutant locus in UA119 was not linked with _fluffy_ and therefore could not be on linkage group II. Moreover, no linkage with _inos_ locus on linkage group V could be detected. It was found unnecessary to test linkage relationships with markers in other linkage groups, since the _fluffy_ isolates from the first _fluffy_ x UA119 cross (Table I) indicated linkage of the mutant locus with _sex_ on linkage group I. It was confirmed by determining the mating type of a large number of ascospore colonies obtained from the second _fluffy_ x UA119 cross. Each isolate was crossed with the wild types 74A and 79a. The data as given in Table III definitely establish the location of the mutant on linkage group I in the vicinity of the _sex_ locus. Among 291 ascospores tested only 14 (4.8%) were possibly recombinants.

TABLE III

Linkage of the "tryptophan" mutation in UA119 with the _sex_ locus.

Ascospores colonies isolated from the cross between UA119 and _fluffy_ were crossed with 74A and 79a to determine the mating type.

Phenotype	ascospores tested	Mating type	
		a	A
Class 1*	121	117	4
Class 2	97	6	91
Class 5#	73	69	4

* Class designations in this table are the same as in Table II.

\# Only vigorously growing _fluffy_ segregants which were known to be prototrophs were tested.

Random ascospore analysis in the absence of other linked markers does not allow determination of the exact location of the "tryptophan" mutation in relation to sex.

The location of the "tryptophan" mutation on linkage group I was confirmed by random ascospore analysis of a cross between UA119 and a leu-3 (leucine requiring, R156) strain. Among 100 viable ascospores, 20 were found to be prototrophs. Leu-3 has been reported to be 9.1 units (tetrad analysis) from sex (Barratt and Ogata, 1954).

II. Recognition of the phen-1 locus as the site of mutation in UA119.

Location of the UA119 mutation in the vicinity of sex suggested that it may be an allele of the phen-1 mutants described earlier by Barratt and Ogata (1954). In fact the possibility would have suggested itself even earlier but the original description of the phen mutants stated that the strains grow vigorously on indole-supplemented medium. UA119, responded very slowly to indole in comparison with tryptophan. The mutant was similar to phen strains in that it did not respond to anthranilic acid. The response of phen-1 strains to indole was noted by Barratt and Ogata in auxanographic tests. However, in the present study the phen strains H6196 and H3791, described by Barratt and Ogata (1954) were found to be similar to UA119 when tested in liquid media supplemented with indole at varying concentrations. The ability to grow when provided with either an aromatic amino acid or leucine (a unique characteristic of phen-1 strains), was displayed

by UA119 and consequently left no doubt that the latter was mutant at the phen-1 locus.

In order to confirm the location of the UA119 mutation at the phen-1 locus the strain was crossed with H6196 and H3791. The fertility of perithecia in these crosses was very low and a very limited number of ascospores was ejected in the cross with H6196; there was no ascospore ejection in the cross with H3791. Ascospores ejected from perithecia of UA119 x H6196 cross were isolated at random. In the case of the UA119 x H3791 cross, perithecia were cut open and ascospores were isolated at random. The data on germination and frequency of prototrophs in these crosses are given in Table IV.

TABLE IV

Segregation of sex and the frequency of prototrophs in crosses of UA119 with phen-1 strains.

Cross	Ascospores analyzed	Germination (%)	Growth	Sex	
				A	a
UA119,A x H6196,a	500	60	wild	208	0
			auxotroph	0	92
UA119,A x H3791,a	600	14.5	wild	0	0
			auxotroph	45	42

While the UA119 x H3791 cross suggested UA119 to be allelic to the phen-1 mutation, the cross UA119 x H6196 was quite anomalous. Fortunately the close linkage of sex to the phen locus afforded an opportunity to analyze the cause of the discrepancy. The possibility was considered that each of the strains, UA119 and H6196, carried one or more suppressors for

the phen-1 allele of the other. In fact the segregation of prototrophs and auxotrophs in the ratio of 2:1 could be explained if one parent had one unlinked suppressor and the other had two unlinked suppressors. The segregation of the sex locus, however, clearly rejected the suppressor hypothesis. The results of the mating-type tests are also given in Table IV. It became clear that the ascospores carrying the sex allele A presumably derived from UA119, were reversions. The high frequency of "phenotypic reversions" (prototrophy due to unspecified genetic changes) in UA119 had been noted earlier. The sample of ascospores analyzed in this case was obviously a biased sample as only those ascospores which had been ejected from perithecia were isolated. It is very likely that the presence of a reverted allele permitted more normal ascus development and behaviour. It should be mentioned that the crossing medium contained lysine because UA119 was also crossed to lysine-3, a linkage group I marker strain and the same medium was employed for all crosses. It was not realized at the time that lysine and arginine have an inhibitory influence on phen-1 strains (Barratt and Ogata 1954). The presence of lysine in the medium might have given the prototrophic nuclei of UA119 a selective advantage.

As the results of the crosses between phen-1 strains and UA119 were not satisfactory it was decided to repeat the crosses, using UA119 and a phen-1 strain reciprocally as the protoperithecial parent. Crosses were made on the synthetic medium of Westergaard and Mitchell (1947) and a Difco corn meal agar (0.2% dextrose); both types of media were supplemented with L-phenylalanine (200 mg./liter). The crosses on

corn meal agar were found to be relatively better in perithecial development and the data given in Table V, on germination percentage and frequency of protrotrophs in ascospore populations, were derived from these crosses. The data are based on samples taken from all the ascospores that could be collected.

It is clear from the data in Table V that the mutant locus in UA119 is allelic to the phen-1 locus. A definite statement about the site of mutation in UA119, in relation to the site or sites involved in H6196 and H3791 is not possible because the nature of the prototrophic isolates was not analyzed; the possibility that the prototrophs are not the products of recombination but due to reversions has to be taken into account.

Table V also shows that the ascospores from each cross yielded an increased number of prototrophic colonies when the plates were incubated for six days. These colonies could be arbitrarily classified into large, medium and small according to their diameter at the time of data collection. It will be seen later, (see reversion frequencies), that a similar situation exists in conidial populations. Microscopic observation of the plates showed that 10-20 percent ascospores germinated and developed mycelial growth to varying extent. It is interesting to note that viability of ascospores on tryptophan-supplemented plates was also in the range of 15-20 percent.

The crosses in which UA119 was the protoperithecial parent yielded fewer prototrophs as compared to the crosses in which UA119 provided the conidia for fertilization. The difference between the two crosses involving UA119 and H6196 is particularly noticeable because in one instance no so called "recombinants" occurred whereas in the other (H6196 x UA119)

TABLE V

Frequency of prototrophs among random ascospores from crosses between UA119 and *phen-1* mutants.

Cross *1	Number of ascospores screened (x 10^5)	Viability (%) *2	Number of viable spores (x 10^4)	Number of "recombinants" *3	"Recombinant" frequencies		Number of proto trophs after 6 days incubation *4		
					% of total spores	% of viable spores	Large	Med.	Small
UA119 x H6196	2.4	16.3	3.90	0	0.000	0.000	11	31	220
UA119 x H3791	5.7	22.1	12.60	6	0.001	0.005	14	67	1000
H6196 x UA119	1.0	14.9	1.49	9	0.009	0.060	9	17	199
H3791 x UA119	1.7	20.0	3.40	26	0.015	0.076	32	19	138

*1 The strain on the left was the protoperithecial parent

*2 Viability estimated from the number of colonies on tryptophan-supplemented plates

*3 "Recombinants" arbitrarily defined as the colonies which were 3 - 5 mm. in diameter on sorbose-minimal agar after 3 days incubation.

*4 Classification based on colony diameter: large 4 mm. or more, medium 2 - 4 mm., small 1 - 2 mm.

nine "recombinants" were found. The ascospore population in the latter case was approximately fifty percent of the former However, the total number of prototrophic colonies, after six days incubation exceeded in the cross UA119 x H6196 when compared to the cross H6196 x UA119.

As the parental strains were not suitably marked for a three point test a satisfactory analysis of the prototrophic progeny could not be undertaken. It was decided that the frequency of "large" prototrophs after three days incubation should give some idea of the frequency of crossing-over between mutant sites in UA119 and the other _phen_-1 strains. Extremely close linkage of the mutant sites is obvious from the data and if it were possible to distinguish revertant and suppressed strains, the frequency of recombinant prototrophs would be perhaps much lower than is apparent from the data.

Among the several prerequisites for a correlative study of gene structure and function are the following:

1. A large number of independently isolated mutants.

2. Suitable outside markers. 3. Criteria for distinguishing the recombinants. 4. Good fertility of inter-allelic crosses and particularly high viability of ascospores. The possible reasons for the apparent scarcity of _phen_-1 mutants are mentioned in General Discussion. Further investigations are required to find out whether or not the prerequisites can be fulfilled. The outside markers would have to be such that the viability of ascospores is not adversely affected. The experience with _fluffy_ indicates that morphological mutants may not be suitable as markers.

III. Effect of different supplements on the viability of conidia of phen-1 mutants.

Barratt and Ogata (1954) studied the relative response of H6196 to growth promoting substances in liquid media and in growth tubes with solidified medium. From their studies it became evident that phenylalanine was the most effective nutrient. In the present study the possibility was considered that differences in viability might also exist at different levels of various supplements added (individually or in combination) to the sorbose-minimal agar medium. The results in Table VI show that there is a significantly higher average number of colonies formed on tryptophan supplemented plates in comparison to those on phenylalanine, leucine or ethyl acetoacetate. Moreover, it can be seen that UA119 and H6196 are similar in behaviour in this respect.

TABLE VI

Frequencies of colonies formed per 100 conidia in the presence of different supplements.

Supplement (1μmole/ml.)	Average number of colonies per 100 conidia	
	UA119	H6196
L-Tryptophan	42.0	53.8
L-Phenylalanine	18.2	29.2
L-Leucine	17.8	21.8
Ethylacetoacetate	21.6	20.4

Since there was a significant difference in the average number of colonies formed on tryptophan medium when compared with other media, it became desirable to know whether or not the differences were restricted to a particular concentration of supplements. Also the response to L-tyrosine was determined. The data obtained are graphically illustrated in Fig. 1. The following conclusions have been deduced from the data: 1. L-tryptophan and L-tyrosine are the most suitable supplements in the sorbose agar medium with regard to viability. On the other hand, there is a sharp reduction in the number of colonies formed in the presence of phenylalanine, leucine or ethyl acetoacetate. 2. There is no significant difference in viability of conidia when germinated on media supplemented with a nutrient except in the case of ethyl acetoacetate. In the latter case, there is an apparent correlation between viability and the concentration of ethyl acetoacetate in the medium. 3. A combination of amino acids in the medium does not increase viability. 4. When compared with other compounds L-tyrosine cause a more rapid development of colonies.

It is not possible, at present, to explain the reduced viability in the presence of phenylalanine, leucine or ethyl acetoacetate. Since L-sorbose has been shown to induce structural changes in the cell wall (Terra and Tatum, 1961), it is possible that the lower viability is a consequence of sorbose in the medium. The effect of sorbose on the permeability mechanisms is not known at present.

Even in the presence of tryptophan or tyrosine only approximately 50% conidia of <u>phen</u>-1 strains give rise to colonies. This reduction in viability may be related to the

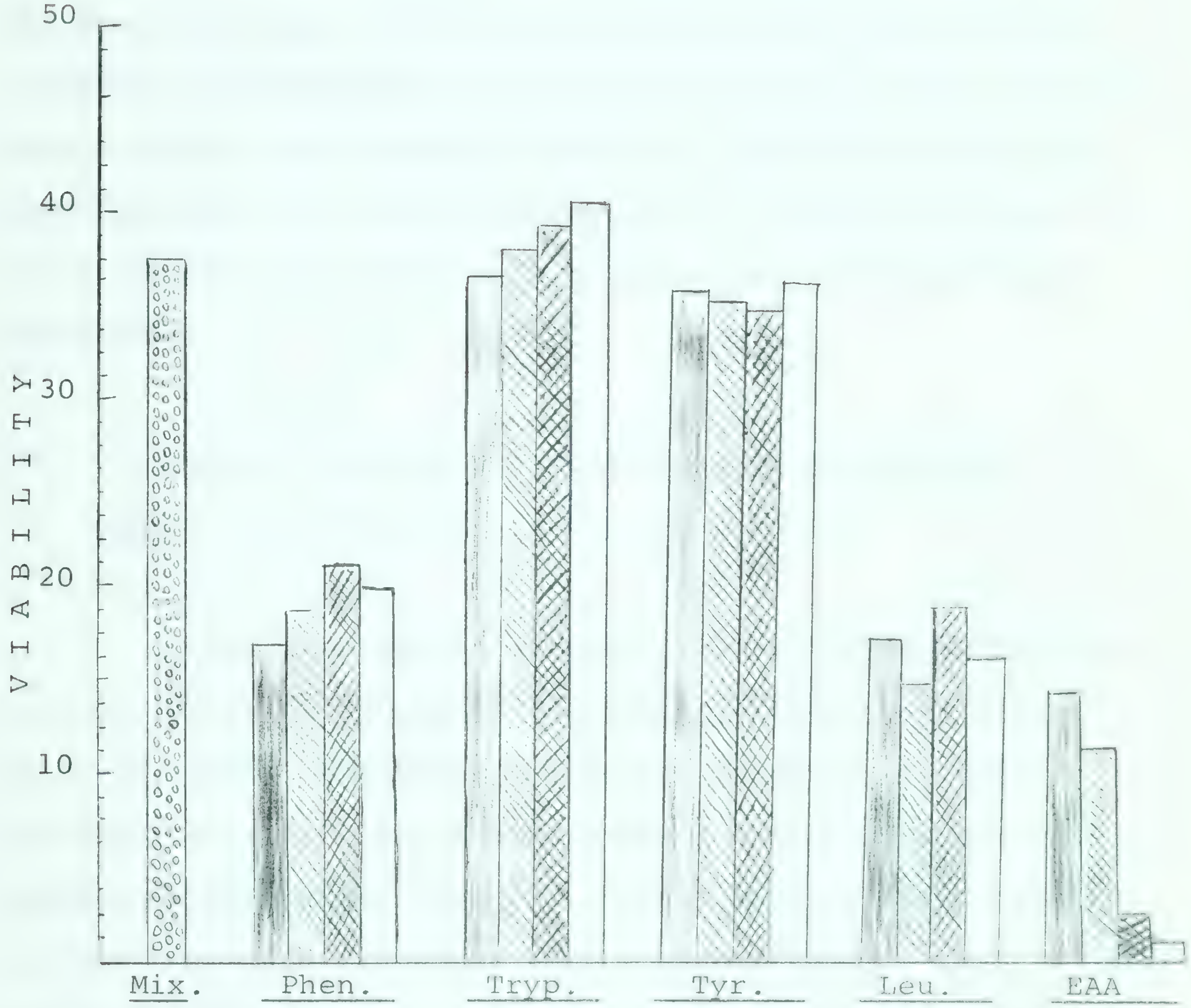

Fig.1. Viability of conidia of UA119 on sorbose-agar media containing various levels of nutrients, singly or in a combination. Viability expressed in number of colonies formed per cent conidia plated. Phen.= L-Phenylalanine: Tryp. = L-Tryptophan; Tyr. = L-Tyrosine: Leu. = L-Leucine: EAA = Ethyl acetoacetate; Mix. = mixture (each amino acid at a concentration of 0.25 μmole per ml. medium)

░ 0.25 μmole/ml. of each amino acid

▒ 1.5 μmole/ml.

▧ 1.0 μmole/ml.

▩ 0.5 μmole/ml.

☐ 0.25 μmole/ml.

frequency of uninuclear microconidia, as it is known that microconidia are more adversely susceptible to environmental changes. A combination of nutrients does not have a synergistic effect on growth of phen-1 mutants (Barratt and Ogata, 1954) and as can be seen in Fig. 1, a combination of amino acids including phenylalanine and leucine does not improve viability when compared with either tyrosine or tryptophan supplement. From the point of view of estimation of viability of conidia it is obvious that tryptophan or tyrosine is the preferred supplement.

IV. Frequency of phenotypic reversions to prototrophy in phen-1 strains.

It has been mentioned earlier that the unusually high rate of reversion is one of the characteristics of UA119 which attracted attention. Reversion frequency is often a characteristic of a particular mutant strain which distinguishes it from other mutants. Furthermore, it is necessary to take reversion frequency into consideration in "interallelic" crosses. Therefore the frequency of "reversions" in UA119 and the other two phen-1 strains have been estimated.

Phenotypic reversion is the conversion of the auxotroph to prototrophy which may be due to mutation within the locus originally mutated or at other sites in the genome; in the latter case the mutational event is called a suppressor mutation and the locus is called a suppressor. In the present study the genetic nature of the revertants has not been investigated. Estimation of reversion frequencies is rather

arbitrary in certain respects. For instance, all the three phen-1 mutant strains are leaky, i.e. conidia of these strains are able to germinate and give rise to minute colonies. Presence of sorbose does not basically alter this characteristic. A certain proportion of conidia are multinucleate and the leakiness may imply an increase in the number of nuclei in each germinating conidium. The frequency of reversion is perhaps best expressed in terms of a population of nuclei but in this study the usual convention of estimation in terms of conidia has been followed.

Due to the "leaky" nature of phen-1 mutants, the conidia of these strains germinate and form minute colonies on minimal sorbose-agar. The colonies are variable in size but the vast majority do not exceed one mm. in diameter after three days incubation at 30 C. It has been assumed that if a colony exceeds one mm. a genetic change is responsible and the colony is regarded as a "revertant". An arbitrary classification of the "revertant" colonies into small (between one and two mm. in diameter), medium (two to four mm.) and large (four mm. or more) was made after incubation of plates for three days. On tryptophan or tyrosine-supplemented plates the colonies attain a diameter of four mm. or more within this period. It was seen that some colonies were compact in appearance with dense growth whereas others were irregular and sparse in growth. This variation in appearance and habit of growth is perhaps significant and reflects variations in genetic nature but the feature was disregarded in the overall classification of reversions.

Table VII gives the frequency of reversions which is

based on the number of "large" and medium" colonies only. The ratio of the two types after three days incubation at 30 C was approximately 1:10 (large:medium). It is obvious that UA119 is relatively much more genetically unstable than H6191 and H3791. A better estimate of relative frequencies will be obtained if more rigorous experimental conditions and criteria are maintained and a larger number of experiments are performed.

TABLE VII

Frequency of reversion in phen-1 strains.

Only the frequency of colonies exceeding two mm. in diameter on sorbose-minimal agar are reported.

Expt.	Mutant	Viability	Number of viable conidia	Number of revertant colonies	Frequency of reversion per 10^7 viable conidia
1	UA119	61.00	6.10×10^6	617	1011.4
2	UA119	41.00	4.10×10^6	416	1014.6
3	UA119	44.00	4.40×10^5	73	1659.1
4	H6196	50.25	5.02×10^6	13	25.79
5	H6196	37.75	3.78×10^6	28	74.07
6	H6196	50.00	5.00×10^6	23	46.00
7	H3791	47.75	4.78×10^6	21	44.93
8	H3791	45.25	4.52×10^6	208	460.17
9	H3791	61.50	6.15×10^6	73	118.59

In all cases the conidial population (10^7 conidia in 1000 ml. medium) has been spread over 70 to 90 plates, except experiment 3 where 10^6 conidia were plated over 10 plates.

The influence of time at which data are collected is illustrated in Fig. 2. It can be seen that the number of "reversions" increases with the length of the incubation period. The difference in relative rates of H3791 in two separate experiments is very striking and illustrates the differences which may exist between samples of the same strain. As far as possible the experimental conditions were kept the same in all respects.

Investigation of the genetic nature of "revertants" would be necessary in order to establish the relative frequency of true revertants (reversions at phen-1 locus) and suppressor mutations. The frequency of fast growing revertants is higher in UA119 and it may be a reflection of the inherent genetic instability of the phen-1 allele in this strain. The higher frequency of phenotypic reversions may also be a consequence of the greater mutability of suppressor gene or genes in UA119 in comparison to those in H6196 and H3791.

The mechanisms of suppression of the phen-1 phenotype can be understood only when the primary action of the mutant phen-1 gene is known. Some of the hypotheses with respect to the nature of phen-1 strains are considered in the General Discussion.

V. "Modified" phen-1 strains obtained from crosses of UA119 with H6196 and H3791.

The crosses between UA119 and other phen-1 strains are interesting from the point of view that they demonstrate the allelic nature of the mutants. But even more interesting

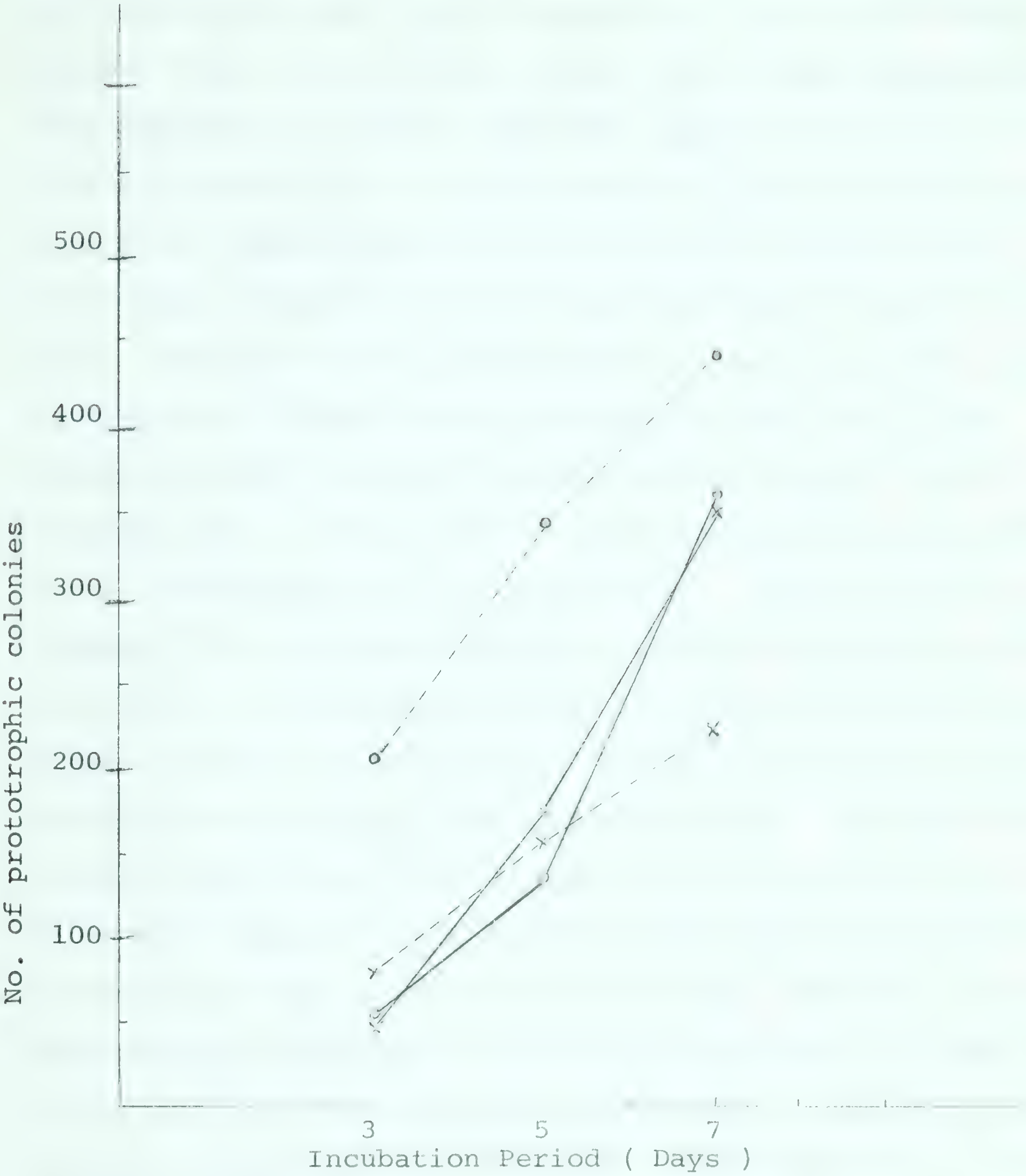

Fig. 2. Increase in frequency of prototrophic colonies with increase in incubation period. x——x & o——o H6196; x---x & o---o H3791.

In each experiment 10^7 conidia were plated in sorbose-minimal agar. Incubation at 30 C.

is the finding that these crosses yield quite a few "segregants" which are modified in their nutritional characteristics. The appearance of these "modified" phen-1 isolates was noticed for the first time in the analysis of a cross between UA119 and H6196 (described earlier in section II of Results). The cross yielded prototrophs and auxotrophs in the ratio of 2:1. The auxotrophic isolates were tested in minimal liquid medium and in liquid media supplemented with one of the compounds which actively promote growth of phen-1 parent strains (Each L-amino acid or ethyl acetoacetate was added at a concentration of 0.5 μmole/ml.). Among 92 strains tested, two were found to be slow growing in minimal medium and none of the compounds had a growth promoting effect on these strains. In contrast to the parental strains, three other strains did not grow on phenylalanine. In addition three strains were found to grow relatively slow on leucine and ethyl acetoacetate media when compared with the parental strains and other progeny from the cross. The most interesting and perhaps significant strains obtained from the cross were the eight which grew only in the presence of leucine (referred to as "leucine specific" strains). It is important to note that these strains did not grow on any of the aromatic amino acids or ethyl acetoacetate. Substitution of glycerol for sucrose as a carbon source did not modify the characteristic. As is the case with the standard wild types, growth on glycerol-supplemented medium was slower but the strains still grew only when provided with leucine and did not grow on other supplemented media. These strains are therefore different from the "modified" phen strains found by Newmeyer (1963).

Acording to Newmeyer, the "slow" phen isolates are inhibited in growth in phenylalanine-supplemented media containing sucrose as a carbon source, but the "slow" strains grow as rapidly as the "fast" in glycerol-medium supplemented with phenylalanine. However, other "modified" strains found in the present investigation may be sensitive to sucrose inhibition.

In the second series of crosses between UA119 and H6196, a total of 194 random ascospores was isolated and tested for growth characteristics on minimal and other media. Two strains grew on minimal medium; nine strains grew slowly on all media though they did not grow on minimal; one strain did not grow on ethyl acetoacetate medium; many other segregants were noticeable for variations in response to the different supplements. From the point of view of the author the main interesting feature was that only one "leucine-specific" strain was found among 194 ascospores sampled.

A total of 277 random ascospores from the two independent crosses between UA119 and H3791 were tested for "modified" strains. No "leucine-specific" strains were found, though many other types of variants were present. Among 190 ascospores isolated from the second cross only one strain grew with wild-type rate on minimal medium but after five days incubation 28 strains showed a fair amount of growth; 33 others could be described as very leaky in comparison with the parents and other ascospore cultures. Since, in qualitative tests the amount of inoculum can not be controlled rigidly the apparent ability to grow in minimal medium at a reduced rate may not be very significant. Moreover, prolonged incubation

in minimal medium would have permitted selective increase of "revertant" nuclei.

The occurrence of eight "modified" strains, which had a specific requirement for leucine, among 92 auxotrophic isolates from the cross between UA119 and H6196 (see Table IV) suggested that these strains resulted from recombination of a number of parental genes. The fact that the above cross also yielded an unexpectedly large number of prototrophs (which was initially suspected to be a consequence of reassortment of genes) lent support to such a point of view. However, the segregation of the closely linked marker sex indicated that the prototrophic isolates were derived from revertant nuclei of UA119. The possibility that the 'leucine-specific' strains originated as a result of recombination of parental genes is moreover unlikely because analysis of a larger population of ascospores from the repeated crosses between UA119 and H6196 yielded only one 'leucine-specific' colony (expected frequency 1.6% of the total population of random ascospores but observed less than 0.1%). It is therefore more plausible that a high mutation rate of the modifying genes in one or the other parent is responsible for the frequent occurrence of the "modified" strains as apparent recombinants. Genetic analysis of the "modified" isolates should give information with regard to the number and location of these 'modifier' genes. It should be pointed out that no 'leucine-specific' strain was isolated among 277 ascospores tested from crosses between UA119 and H3791. This fact indicates the possible allele specificity of the modifying gene or genes concerned.

The term "modifier" is used to qualify a large number of genes whose presence becomes known only because of their interaction with other genes with more easily identifiable effects. Certain mutations in the broad category of "modifier genes" are recognized because they suppress the phenotypic expression of other genes. Suppressor mutations affecting genes whose nature and mode of action is well understood have received considerable attention and in many cases reasonable explanations with respect to the biochemical activity of "suppressor" genes are available. However, other "modifier genes", like those present in *nt* strains (Haskins and Mitchell, 1952; Newmeyer and Tatum, 1954) and those affecting the *phen*-1 phenotype are not understood because the action of the "major" gene is not known.

In the case of "modified" *phen*-1 strains, the genetic and physiological mechanisms involved in their phenotypic expression may be significant with respect to the primary role of the *phen*-1 gene. We may consider, for example, the "modified" strains which do not grow in phenylalanine-supplemented medium and a few others which grow only in the presence of leucine. Inhibition of growth in phenylalanine-supplemented medium could either be due to a constituent of the medium or alternatively phenylalanine does not play the same role in the "modified" isolates as it does in the parental strains.

The strains which have an obligatory requirement for leucine also raise many questions. A specific requirement for leucine in the "modified" strain could be due to a genetic block in the biosynthesis of leucine without altera-

tion of the primary action of the phen-1 gene. However, if the above assumption is incorrect, any hypothesis to explain the nature and mode of action of mutant phen-1 gene, must be somehow reconciled with the existence of the "modified" phen-1 strains.

VI. Nutritional characteristics of the phen-1 mutants and their derivatives.

Since Barratt and Ogata (1954) have given extensive data with regard to the nutritional characteristics of a phen-1 mutant (H6196) to different supplements and UA119 was found to be similar to H6196, no detailed quantitative study of growth response of UA119 and the other phen-1 was carried out. The preliminary results obtained in the present study confirmed the observations of Barratt and Ogata (loc. cit.) with the following exceptions:

1. phen-1 mutants were not found to grow in the presence of indolepyruvic acid at any concentration in a wide range (this finding seems important when considering the biochemical nature of the phen-1 mutants).

2. On the basis of auxanographic tests Barratt and Ogata reported that indole promoted active growth of phen-1 mutants. This statement needs further qualification because the response of phen-1 mutants to indole was found to be considerably less than response to other growth promoting compounds. Concentrations of indole in excess of 0.25 µmole/ml. were inhibitory, just as it has been known to be inhibitory in other types of tryptophan mutants which respond to indole.

In a typical test using stationary cultures, phen-1 mutants produce 40-50 mg dry matter in 72 hrs. of incubation at a L-tryptophan concentration of 0.25 μmole/ml. medium. When grown in indole medium (0.25 μmole indole/ml.) the dry matter produced is less than 20 mg. after 92 hrs. incubation.

3. Phenylpyruvic acid (the α-keto precursor of phenylalanine) promotes growth of phen-1 mutants even less than indole. The α-keto precursor of tyrosine, p-hydroxyphenylpyruvic acid, is less effective than phenylpyruvic acid.

4. A combination of indole, phenylpyruvic acid and p-hydroxyphenylpyruvic acid, each at a concentration of 0.25 μmole/ml., is more effective (approximately 50% more) than indole alone. Higher concentrations of phenylpyruvic acid and p-hydroxyphenylpyruvic acid are inhibitory.

5. The α-keto precursor of leucine, α-keto-isocaproic acid, is inactive for growth promotion of phen-1 mutants when tested over a wide range of concentrations.

GENERAL DISCUSSION

Discovery of a new phen-1 mutant revives interest in the study of the somewhat unorthodox peculiarities of these mutants. Possibly, the study of phen-1 mutants may have a wider significance with respect to the concepts of gene action but certainly, the consequences of mutations at phen-1 locus, as reflected in the nutritional characteristics of the mutants, warrant an extensive genetic and biochemical study. The most important genetic question posed by phen-1 mutants is whether or not the mutations causing phen-1 phenotype are restricted to a certain region within the functional unit (the gene). Biochemically, the obvious problem is to explain the nutritional characteristics of phen-1 mutants in enzymatic or some other molecular terms.

Mutants at the phen-1 locus are conspicuous because of their rare occurrence. The reasons for the apparent scarcity of phen-1 mutants could be several, though the ability to grow in the presence of any one of a large number of compounds would suggest much more frequent detection of such mutants.

The most obvious reason for the scarcity seems to be that efforts are usually directed to selection of mutations at a specific locus and hence the strains which are not mutant at that particular locus are discarded. Moreover discrimination against the "leaky" strains may constitute a selective disadvantage for the phen-1 mutants.

Selection of nutritional mutants is usually made with one specific compound and the number of genes affecting the biosynthesis of the compound will influence the frequency

with which a mutation at any specific locus is isolated. Moreover, the probability for mutations is not the same for all loci. For instance, a mutation at any one of six different loci in Neurospora creates a nutritional requirement which is satisfied by tryptophan, whereas auxotrophs requiring leucine are known to occur as a result of mutation at any one of five loci. Consequently the best selective compound for phen mutants is ethyl acetoacetate as no other mutants are known to respond to this compound.

Filtration enrichment technique (Woodward, DeZeeuw and Srb, 1954) is most commonly employed for selection of nutritional mutants and the mutants which are leaky would probably be eliminated in this technique. The phen mutant UA119 was isolated by the "inositol-less death" technique of Lester and Gross (1961) and leaky mutants are not discriminated against in this procedure. The mutants H6196 and H3791 were isolated by Hungate and Mannell (1952) without the use of any enrichment step.

The most interesting possibility, however, is that the phen locus controls a function which can be rescued by exogenous supply of certain compounds only when the mutation occurs in a specific region of the gene; mutations at other sites in the locus may either create no nutritional requirement or their growth inhibitory effects may not be reversed by one or several compounds. This possibility becomes quite significant if a transaminase is implicated in phen mutations. The transaminase hypothesis is considered later in this discussion.

It is difficult to propose a satisfactory hypothesis

to explain the nutritional requirements of phen-1 mutants because information with respect to the metabolism of relevant compounds in *Neurospora* (particularly the degradative pathways) is incomplete. Moreover, the specific mechanisms involved in the regulation of metabolic pathways in *Neurospora* are as yet not sufficiently explored.

Barratt *et al.*, (1956) tested the possibility that mutation in *phen*-1 gene blocks the biosynthesis of phenylalanine and leucine and that exogenous phenylalanine and leucine are interconverted via acetoacetate. The results of this study showed, however, that the pathways of phenylalanine and leucine were not linked. It is now known that the intermediates involved in the biosynthesis of leucine are different from the intermediates in the biosynthetic pathways of the aromatic amino acids. Consequently the models derived from studies of other mutants whose growth is promoted by more than one amino acid (see Review) cannot be applied to *phen*-1 mutants.

Barratt *et al.*, (1956) suggested the possibility that the *phen*-1 mutants are unable to synthesize an essential compound, which is either very labile or exists in very minute amounts. The hypothetical requirement could, however, be synthesized by the organism by utilization of specific exogenous compounds. These authors found radioactive label from both, phenylalanine and leucine, in some ninhydrin-negative compounds and suggested that an investigation of these latter compounds may give a clue to the nature of the assumed essential compound. It is difficult to imagine that the deficiency of such a compound could be the primary effect

of the mutant gene. If the biosynthetic pathways of phenylalanine and leucine are operating normally and the intracellular concentration of one or the other amino acid is adequate a deficiency of the hypothetical compound could hardly occur. In addition, such a proposed mechanism should be able to explain the growth-promoting ability of tryptophan, kynurenine, norleucine etc.

Another possibility suggested by Barratt _et al_.,(1956) considers accumulation of a growth inhibitor as a result of the primary metabolic block in the _phen_-1 strain. This hypothesis, however, raises a number of questions concerning the nature of the primary metabolic block, the nature of the inhibitor, the reasons for the specificity of the inhibitor for certain compounds and the action of the inhibitor in relation to the metabolism of the organism. It should be noted that the hypothesis does not consider the primary metabolic block as the cause of growth inhibition and therefore regards the product or products of this reaction as unessential. Experimental proof for the existence of such an inhibitor is necessary and perhaps it may be obtained if the inhibitor is a stable, small molecule which is accumulated in the culture fluid. The inhibitor could be recognized by its effect on non-mutant strains provided it is not excluded by permeability factors. If the inhibitor remains intracellular, however, it might not be easy to demonstrate its existence. Since variations of pH or the ionic composition of the minimal medium do not alter the auxotrophic nature of _phen_-1 mutants (Barratt and Ogata, 1954), the inhibitor, if it exists, is not affected by these factors. It is known

that amino acid analogues such as DL-norleucine and p-fluorophenylalanine promote the growth of phen-1 strains (Barratt and Ogata, 1954; A. Gib De Busk, personal communication). Since analogues can often substitute for the normal amino acid in many reactions and can generally inhibit the growth of the organism, the presence of an inhibitor in phen-1 mutants is not necessarily indicated.

It seems profitable to consider the possibility that the activity of a transaminase is modified due to mutation at the phen-1 locus. A transamination reaction is the final step in the biosynthesis of phenylalanine, tyrosine and leucine and the conversion to the α-keto acid is the first step in the degradation of tyrosine and leucine. Degradation of phenylalanine can take place through its conversion to tyrosine or may involve its conversion to phenylpyruvic acid. The "transaminase hypothesis" proposes that the phen-1 mutation causes a reduction in the biosynthesis of phenylalanine, tyrosine and leucine due to modification of the transaminase. This modification could alter the affinity of the enzyme for certain amino group donors in the transamination reactions with α-keto precursors of phenylalanine, tryosine and leucine and consequently reduce the rate of synthesis of these amino acids. It may be, that in the phen-1 mutants an exogenous amino acid which promotes the growth of these strains, is preferentially utilized for the amination of the α-keto precursors of certain other amino acids. Since biosynthesis of tryptophan does not involve a transamination reaction, the ability of tryptophan to promote growth of phen-1 mutants may either depend on its

effect on the phenylalanine and tyrosine pathways or its role as an amino group donor in transamination reactions. Exogenous tryptophan may inhibit and repress the tryptophan pathway and lead to an increased rate of synthesis of phenylalanine and tyrosine. Kynurenine also promotes growth of phen-1 mutants and may be an amino group donor in transamination reactions. Wild-type Neurospora possesses a transaminase which catalyzes amination of a number of α-keto acids with kynurenine as the amino group donor (Jacoby and Bonner, 1956). Because anthranilic acid does not stimulate growth of phen-1 mutants, the conversion of kynurenine to anthranilic acid could not be an important reaction. Indole, phenylpyruvic acid, p-hydroxyphenylpyruvic acid and other substances which stimulate growth of phen-1 mutants to varying extent are probably active due to indirect influence on the metabolism of phen-1 strains. It is possible, however, that some of these compounds directly affect the transaminase reactions. Acetoacetate may be assumed to serve a regulatory function and act by reducing the degradation of tyrosine (phenylalanine) and/or leucine. It is also possible that a compound derived from acetoacetate, for example β-hydroxy-β-methylglutaryl CoA, exerts a direct influence on the metabolism of leucine.

The inability of the α-keto precursors of phenylalanine, tyrosine and leucine to promote vigorous growth of phen-1 mutants is the principal reason for considering the "transaminase hypothesis". The immediate precursor of leucine, α-ketoisocaproate, does not stimulate growth at all and phenylpyruvic acid and p-hydroxyphenylpyruvic acid, in comparison to phenylalanine and tyrosine, are poor supplements

for the growth of mutants. Strains of Neurospora blocked at an earlier step in the biosynthetic pathway of phenylalanine or leucine are known to utilize the corresponding α-keto compound (Metzenberg and Mitchell, 1956; Regnery, 1944). It should be noted, however, that many isoleucine-valine mutants of Neurospora are known to possess the necessary transaminase but do not grow when provided with the α-keto precursors of isoleucine and valine (Wagner et al., 1964). Therefore slow or no growth in the presence of a α-keto acid is not a decisive evidence in favour of the transaminase hypothesis.

The transaminase hypothesis would have to be supported by investigations in 'modified' phen-1 strains. In this respect the "leucine-specific" derivatives of phen-1 mutants should be particularly helpful. If the altered behaviour of a strain is not due to a genetic change in the phen-1 allele, the properties of the transaminase in the 'modified' strain should be the same as in the parent phen-1 mutants. In addition, the phenotype of the 'modified' strain should be explicable on the basis of its other metabolic or physiological characteristics.

Serious objections to the transaminase hypothesis arise due to the inability of isoleucine and valine to promote growth of phen-1 strains. Studies of transamination activities in Neurospora indicate that a single enzyme catalyzes the transamination reactions involving phenylalanine, tyrosine, leucine, isoleucine and valine (Fincham and Boulter, 1956; Seecof and Wagner 1959a,b,). A transaminase which catalyzed readily transamination between phenylalanine, isoleucine, valine or leucine and the α-keto analogues of the amino

acids, was partially purified by Seecof and Wagner (1959a,b). It is interesting to note that this transaminase had an extremely high affinity for phenylpyruvate. The preparation readily transaminated α-ketoglutarate with phenylalanine as an amino group donor but the reaction between glutamate and phenylpyruvate was extremely slow. If a single transaminase catalyzes reactions involving phenylalanine, tryosine, leucine, isoleucine and valine, then the hypothesis requires that isoleucine and valine be ineffective as amino group donors for amination of one or all α-keto precursors of leucine, phenylalanine and tyrosine.

Investigation of the mechanism involved in the inhibition of phen-1 strains, when arginine or lysine is present in the medium, may be useful. Most often the cause of inhibition of a mutant strain is the inadequate uptake of the essential nutrient due to the presence of certain other compounds. For example, a tryptophan-requiring mutant of Neurospora was inhibited by fourteen different amino acids and phenylalanine, leucine, methionine, tyrosine and norleucine were the most effective inhibitors (Brockman, 1964). It is interesting to note that arginine and lysine have been found to be non-inhibitory for mutants requiring an aromatic amino acid and the phen-1 mutants are distinctive in this respect. If inhibition is not due to permeability factors, the phenomenon may have significance with respect to the primary effect of the phen-1 mutant gene.

It is reasonable to consider that properties of the transaminase would be modified due to certain mutations. Mutations could also cause a complete loss of the enzyme

activity but the consequences might be irreparable. Since there is no information about genetic control of transaminases in Neurospora, it is perhaps more attractive to postulate that certain mutations in the phen-1 gene cause production of altered enzyme molecules which are functional under certain intracellular conditions. A knowledge of the composition and other characteristics of the so called "pool" of free amino acids and α-keto acids would be particularly useful in an experimental evaluation of the transaminase hypothesis. The properties of the enzyme and the characteristics of the "pool" would determine the in vivo role of the transaminase.

The basic source of amino groups in Neurospora crassa is glutamate which is synthesized from α-ketoglutarate and ammonium ions by glutamic dehydrogenases. While glutamate undoubtedly plays a central role in the amino acid metabolism of the organism and is generally present in high concentrations in the mycelium, it may not be utilized for certain transamination reactions to the same extent as for some others.

Phen-1 mutants highlight the need for studies of phenylalanine, tyrosine and leucine metabolism in Neurospora, particularly in relation to acetoacetate. It need not be emphasized that a knowledge of the endogenous synthesis and fate of the aromatic and the branched-chain amino acids and specially the related α-keto acids would be essential if the phen-1 mutants are to be understood. The hypothesis that certain mutations in the phen-1 locus result in alterations of the properties of a transaminase with the consequent

nutritional characteristics, seems to be plausible in spite of numerous difficulties at present. It is amenable to experimental evaluation and in any case, studies based on this hypothesis should lead to a better understanding of the unorthodox behaviour of _phen_-1 mutants. A trivial outcome of these studies is likely to be a more appropriate designation for the mutants because "phen" is certainly inadequate and rather misleading.

SUMMARY AND CONCLUSIONS

1. A new phen-1 mutant has been discovered as a result of genetic analysis of the strain UA119. The strain was isolated as a tryptophan-requiring auxotroph and was earlier reported to be a tryp-3 (td) mutant. Close linkage to the sex locus and very low frequency of prototrophs in large populations of ascospores from crosses between UA119 and two previously described phen-1 mutants, establish UA119 as a phen-1 mutant.

2. The frequency of apparent recombinants (prototrophs) in "inter-allelic" phen-1 crosses varies between 0 and 0.015% total random ascospores. The variation is believed to be, at least partly, due to the genetic instability of phen-1 mutants, particularly UA119.

3. The frequency of phenotypic reversions in UA119 (approx. $10/10^5$ viable conidia; only colonies 2 mm. or more in diameter counted; sorbose-agar medium; incubation 3 days at 30 C) is significantly higher than in the other phen-1 mutants, H6196 and H3791 (the range in the latter two was between 0.26 and 4.60 per 10^5 viable conidia). The "large" prototrophs (4 mm. or more) are approximately 10% of total revertants.

4. Probably mutations at the phen-1 locus as well as other "suppressor" mutations are responsible for the phenotypic reversions. The high frequency of reversions in UA119 may be due to the specific phen-1 allele and the genetic background.

5. The viability of conidia of phen-1 mutants on L-tryptophan- or L-tyrosine-supplemented medium (sorbose-agar)

varies between 40 - 60%. However, the media supplemented with L-phenylalanine, L-leucine or ethyl acetoacetate reduce the viability to 20% or less. The physiologic effects of sorbose may be involved in the reduction of viability in the presence of the latter compounds.

6. The viability of ascospores from "inter-allelic" crosses is very low (approx. 15-20%) and prototrophs and "modified" strains are frequent among the progeny. Prototrophy appears to enhance the viability of ascospores.

7. "Modified" isolates are recognized by their inability to grow on minimal medium and media supplemented with any one compound which promote growth of the parental phen-1 strains. The "phenylalanine-negative" and "leucine-specific" strains (the former do not grow in phenylalanine-supplemented medium and the latter have a specific requirement for leucine) are particularly interesting. The genetic and biochemical aspects of the "modified" strains remains to be investigated.

8. The reasons for the apparent scarcity of phen-1 mutants are discussed. It is suggested that ethyl acetoacetate is the best selective compound for phen-1 mutants.

9. Various hypotheses proposed to explain the nutritional characteristics of the phen-1 mutants are discussed. A new hypothesis is suggested which postulates that certain mutations at the phen-1 locus lead to an altered transaminase. The altered enzyme is unable to catalyze transamination reactions involved in the biosynthetic and other metabolic pathways of phenylalanine, tyrosine and leucine. The hypothesis is attractive because it is relatively more amenable to experimental tests.

BIBLIOGRAPHY

Ames, B.N. and Horecker, B.L. 1956. The biosynthesis of Histidine: Imidazoleacetol Phosphate Transaminase. J.Biol. Chem. 220: 113-128.

Barratt, R.W. and Ogata, W.N. 1954. A strain of Neurospora with an alternative requirement for leucine or aromatic amino acids. Am. J. Botany 41: 763-771.

Barratt, R.W., Fuller, R.C. and Tanenbaum S.W. 1956. Amino acid interrelationships in certain leucine and aromatic-requiring strains of Neurospora crassa. J. Bacteriol. 71: 108-114.

Barratt, R.W., Newmeyer, D., Perkins, D.D. and Garnjobst, L. 1954. Map Construction in Neurospora crassa. Advan. Genet. 6: 1-93

Beadle, G.W. 1945. Biochemical Genetics. Chem. Rev. 37: 15-96.

Beadle, G.W. 1959. Genes and chemical reactions in Neurospora. Science 129: 1715-1719.

Beadle, G.W. and Tatum E.L. 1941. Genetic control of biochemical reactions in Neurospora. Proc. Nat. Acad. Sci. U.S. 27: 499-506.

Bonner, D.M., Yanofsky, C. and Partridge, C.W.H. 1952. Incomplete genetic blocks in biochemical mutants of Neurospora. Proc. Nat. Acad. Sci. U.A. 38: 25-34.

Bonner, D.M. and Yanofsky, C. 1951. The biosyntheses of tryptophan and niacin and their relationships. J. Nutrition 44: 603-616.

Braithwaite, G.D. and Goodwin, T.W. 1960. Incorporation of (^{14}C) acetate, (^{14}C) mevalonate and $^{14}CO_2$ into β-carotene by a fungus Phycomyces blakesleeanus. Biochem. J. 76: 5-10.

Brockman, H.E. 1964. Effects of amino acids on the utilization of tryptophan and indole for growth by a mutant of Neurospora crassa. J.Gen. Microbiol. 34: 31-41.

Burns, R.O., Umbarger, H.E. and Gross, S.R. 1963. Biosynthesis of leucine III. The conversion of α-hydroxy-β-carboxyisocaproate to α-ketoisocaproate. Biochem. 2: 1053-1058.

Calvo, J.M., Kalyanpur, M.G. and Stevens, C.M. 1962. 2-Isopropylmalate and 3-isopropylmalate as intermediates in leucine biosynthesis. Biochem. 1: 1157-1161.

Coon, M.J., Robinson, W.G. and Bachhawat, B.K. 1955. Enzymatic studies on the biological degradation of the branched chain amino acids. p. 431-441 In McElroy, W.D. and Glass, H.B. (Eds.). Amino Acid Metabolism. The Johns Hopkins Press, Baltimore.

Davis, R.H. 1962. Consequences of a suppressor gene effective with pyrimidine and proline mutants of *Neurospora*. Genetics 47: 351-360.

Davis, R.H. and Thwaites, W.M. 1963. Structural gene for ornithine transcarbamylase in *Neurospora*. Genetics 48: 1551-1558.

Durr, I.F. and Rudney, H. 1960. Reduction of β-hydroxy-β-methylglutaryl coenzyme A to mevalonic acid. J. Biol. Chem. 235: 2572-2578.

Emerson, S. 1950. Competitive reactions and antagonisms in the biosynthesis of amino acids by *Neurospora*. Cold Spring Harbor Symp. Quant. Biol. 14: 40-48.

Fincham, J.R.S. 1960. Genetically controlled differences in enzyme activity. Advan. Enzymol. 22: 1-43.

Fincham, J.R.S. and Boulter, A.B. 1956. Effects of amino acids on transaminase production in *Neurospora crassa*: evidence for four different enzymes. Biochem. J. 62: 72-77.

Garrod, A.E. 1909. Inborn errors of metabolism. Oxford University Press, London.

Gibson, F. 1964. Chorismic acid: purification and some chemical and physical studies. Biochem. J. 90: 256-260.

Gibson, F., Gibson, M. and Cox G.B. 1964. The biosynthesis of p-aminobenzoic acid from chorismic acid. Biochem. Biophys. Acta. 82: 636-639.

Gibson, F. and Jackman, L.M. 1963. Structure of chorismic acid, a new intermediate in aromatic biosynthesis. Nature 198: 388-389.

Gibson, M.I. and Gibson, F. 1964. Preliminary studies on the isolation and metabolism of an intermediate in aromatic biosynthesis: chorismic acid. Biochem. J. 90: 248-255.

Green, D.E. and Wakil, S.J. 1960. Enzymatic mechanisms of fatty acid oxidation and synthesis. Pages 1-40 *in* Bloch K (Ed.) Lipide Metabolism. John Wiley and Sons, New York and London.

Gross, S.R., Burns, R.O. and Umbarger, H.E. 1963. Leucine biosynthesis II. The enzymatic isomerization of β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate. *Biochem*. *2*: 1046-1052.

Gross, S.R. and Fein, A. 1960. Linkage and function in *Neurospora*. Genetics *45*: 885-904.

Haskins, F.A. and Mitchell, H.K. 1948. Evidence for a tryptophan cycle in *Neurospora*. Proc. Nat. Acad. Sci. U.S. *35*: 500-506.

Haskins, F.A. and Mitchell, H.K. 1952. An example of the influence of modifying genes in *Neurospora*. Am. Naturalist *86*: 231-238.

Henderson, L.M., Gholson, R.K. and Dalgliesh, C.E. 1962. Metabolism of aromatic amino acids. Pages 245-342 in Florkin, M. and Mason, H.S. (Eds.) Comparative Biochemistry. Vol. IV. Academic Press, New York and London.

Hungate, R.P. and Mannell, T.J. 1952. Sulphur - 35 as a mutagenic agent in *Neurospora*. Genetics *37*: 709-719.

Jacob, F. and Monod, J. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. *3*: 318-356.

Jacoby, W.B. and Bonner, D.M. 1956. Kynurenine transaminase from *Neurospora*. J. Biol. Chem. *221*: 689-695.

Jungwirth, C., Gross, S.R., Margolin, P. and Umbarger, H.E. 1963. The biosynthesis of leucine I. The accumulation of β-carboxy-β-hydroxyisocaproate by leucine auxotrophs of *Salmonella typhimurium* and *Neurospora crassa*. Biochem. *2*: 1-6.

Leeuw, A. de 1963. The mutagenic action of nitrous acid and ultraviolet light on conidia of *Neurospora crassa*. M. Sc. Thesis, Univ. of Alberta.

Lester, G. 1963. Regulation of early reactions in the biosynthesis of tryptophan in *Neurospora crassa*. J. Bacteriol. *85*: 468-475.

Lester, H.E. and Gross, S.R. 1961. Efficient method for selection of auxotrophic mutants in *Neurospora*. Science *129*: 572.

Metzenberg, R.L. and Mitchell, H.K. 1956. Isolation of prephenic acid from *Neurospora*. Arch. Biochem. Biophys. *64*: 51-56.

Metzenberg, R.L. and Mitchell, H.K. 1958. The biosynthesis of aromatic compounds by *Neurospora crassa*. Biochem. J. *68*: 168-172.

Mitchell, H.K. and Lein, J. 1948. A *Neurospora* mutant deficient in the enzymatic synthesis of tryptophan. J. Biol. Chem. *175*: 481-482.

Mitchell, M.B. and Mitchell, H.K. 1952. Observations on the behaviour of suppressors in *Neurospora*. Proc. Nat. Acad. Sci. U.S. *38*: 205-214.

Monder, C. and Meister, A. 1958. α-Ketoglutaramic acid as a product of enzymic transamination of glutamine in *Neurospora*. Biochim. Biophys. Acta *28*: 202-203.

Morgan, P.M., Gibson, M.I. and Gibson, F. 1963. The conversion of shikimic acid into certain aromatic compounds by cell-free extracts of *Aerobacter aerogenes* and *Escherichia coli*. Biochem. J. *89*: 229-239.

Newmeyer, D. 1963. Altered phenotype of *phen*. Neurospora Newsletter No. 4, page 10.

Newmeyer, D. and Tatum, E.L. 1953. Gene expression in *Neurospora* mutants requiring nicotinic acid or tryptophan. Am. J. Botany *40*: 393-400.

Partridge, C.W.H., Bonner, D.M. and Yanofsky, C. 1952. A quantitative study of the relationship between tryptophan and niacin in *Neurospora*. J. Biol. Chem. *194*: 269-278.

Popják, G. and Cornforth, J.W. 1960. Biosynthesis of cholesterol. Advan. Enzymol. *22*: 281-335.

Regnery, D.C. 1944. A leucine-less mutant strain of *Neurospora crassa*. J. Biol. Chem. *154*: 151-160.

Rudman, D. and Meister, A. 1953. Transamination in *Escherichia coli*. J. Biol. Chem. *200*: 591-604.

Sarabhai, A.S., Stretton, A.O.W., Brenner, S. and Bolle, A. 1964. Co-linearity of the gene with the polypeptide chain. Nature *201*: 13-17.

Seecof, R.L. and Wagner, R.P. 1959a. Transaminase activity in *Neurospora crassa*. I. Purification and substrate specificity of a phenylpyruvate transaminase. J. Biol. Chem. *231*: 2689-2693.

Seecof, R.L. and Wagner, R.P. 1959b. Transaminase activity in Neurospora crassa. II. Kinetic behaviour of a phenylpyruvate transaminase. J. Biol. Chem. 234: 2694-2697.

Srb, A.M. and Horowitz, N.H. 1944. The ornithine cycle in Neurospora and its genetic control. J. Biol. Chem. 154: 129-139.

Stent, G.S. 1964. The operon: on its third anniversary. Science 144: 816-820.

Tatum, E.L. 1946. Induced biochemical mutations in bacteria. Cold Spring Harbor Symp. Quant. Biol. 11: 278-284.

Tatum, E.L., Barratt, R.W. and Cutter, V.M. 1949. Chemical induction of colonial paramorphs in Neurospora and Syncephalastrum. Science 109: 509-511.

Terra, Noel de, and Tatum, E.L. 1961. Colonial growth of Neurospora. Science 134: 1066-1068.

Umbarger, H.E. 1956. Evidence for a negative feedback mechanism in the biosynthesis of isoleucine. Science 123: 848.

Umbarger, H.E. 1961. Feedback control by endproduct inhibition. Cold Spring Harbor Symp. Quant. Biol. 26: 301-312.

Umbarger, H.E. and Davis, B.D. 1961. Intermediates in amino acid biosynthesis. P. 167-251. In I.C. Gunsalus and R.Y. Stanier (ed.), The Bacteria Vol. III Biosynthesis. Academic Press, New York.

Vogel, H.J. 1955. On the glutamate-proline-ornithine interrelation in various micro-organisms. P. 335-346. In W.D. McElroy and H.B. Glass (ed.) Amino Acid Metabolism. The Johns Hopkins Press, Baltimore.

Vogel, H.J. 1956. A convenient growth medium for Neurospora crassa. Microbial Genetics Bull. No. 13: 42-43.

Vogel, H.J. 1961. Control by repression. P. 23-65. In D.M. Bonner (ed.), Control Mechanisms in Cellular Processes. Ronald Press Co., New York.

Vogel, R.H. and Kopac, M.J. 1959. Glutamic γ-semialdehyde in arginine and proline synthesis of Neurospora: a mutant-tracer analysis. Biochim. Biophys. Acta 36: 505-510.

Wagner, R.P. and Mitchell, H.K. 1964. Genetics and Metabolism. 2nd edition. John Wiley & Sons, Inc., New York.

Wagner, R.P., Bergquist, A., Barbee, T. and Kiritani, K. 1964. Genetic blocks in the isoleucine-valine pathway of Neurospora crassa. Genetics 49: 865-882.

Westergaard, M. and Mitchell, H.K. 1947. Neurospora. V. A synthetic medium favoring sexual reproduction. Am. J. Botany 34: 573-577.

Woodward, V. W., De Zeeuw, J.R. and Srb, A.M. 1954. The separation and isolation of particular biochemical mutants of Neurospora by differential germination of conidia, followed by filteration and selective plating. Proc. Nat. Acad. Sci. U.S. 40: 192-200.

Yanofsky, C., Helinski, D.R. and Maling, B.D. 1961. The effects of mutation on the composition and properties of the A protein of Escherichia coli Tryptophan Synthetase. Cold Spring Harbor Symp. Quant. Biol. 26: 11-24.

Yanofsky, C., Carlton, B.C., Guest, J.R., Helinski, D.R. and Henning, U. 1964. On the colinearity of gene structure and protein structure. Proc. Nat. Acad. Sci. U.S. 51: 266-272.